IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of
WANG et al.
Appln. No. 10/570,505
Filed: June 5, 2006

Confirmation No. 2905
Atty. Ref.: 1328-28
T.C. / Art Unit: 1657
Examiner: R. J. GITOMER

FOR: METHOD FOR DETERMINING THE IMPACT OF A MULTICOMPONENT NATURAL PRODUCT MIXTURE

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April 12, 2011

Mail Stop AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450
Sir:

Rule 132 Declaration of Dr. Jan van der Greef

I, **Dr. Jan van der Greef** of **De Beaufortlaan 8, 3971 BM Driebergen, The Netherlands**, declare the following:

1. I earned a PhD degree in 1980 from the Amsterdam University.
2. I have been employed by TNO since 1980.
3. I currently hold the position of TNO Principal Scientist and my duties include research and development in life sciences especailly related to pharma, nutrition, natural products and herbal medicine.
4. I am a co-inventor of the inventions claimed in this patent application.
5. I have read and I am familiar with the above referenced patent application (U.S. Appl. No. 10/570,505), the Office Action of November 12, 2010 and the cited

references Huyn (U.S. Publication 2002/0095260), Borisy (U.S. Publication 2003/0096309), Afeyan (U.S. Publication 2005/0283320), Khwaja (U.S. Patent 6,379,714) and Pugh (J. Agricultural Food Chem). I have read and am familiar with the current pending claims in the above referenced patent application (U.S. Appl. No. 10/570,505).

6. I have reviewed the Office Action of November 12, 2010. I understand that claims 1-20 and 25 are pending. I understand that claims 1-20 and 25 stand rejected under 35 U.S.C. 103 as allegedly obvious in view of Huyn (U.S. Publication 2002/0095260), Borisy (U.S. Publication 2003/0096309), Afeyan (U.S. Publication 2005/0283320), Khwaja (U.S. Patent 6,379,714) or Pugh (J. Agricultural Food Chem). I also understand that 1-20 and 25 stand rejected under 35 U.S.C. 112, first paragraph.

7. In the Office Action, the Examiner referred to the Section 112 requirements and expressed an opinion that the specification does not enable one of skill in the art how to make and use the claimed methods. I wish to provide the Examiner with the following comments and experimental data to show that one of skill in the art would know how to practice the features of the claims and would be enabled by this Specification to practice, make and use the full scope of the claims.

8. Using the disclosure of the Specification, I performed an experiment to discover synergistic compounds in extracts of cannabis. The objective of the experiment

was to find compounds in extracts of cannabis that potentially have a beneficial effect in relation to curing certain diseases. In this case, it is known that inflammation is a core issue in many diseases in living systems, for that reason a cell based inflammation system was used to determine a biological profile of a disease. The biological profile can be determined by comparing the activity and distribution of human monocytes in normal and diseased individual. Methods for such comparison are commonly known. However, correlating experimental results with activities of chemical compounds in a complex mixture have not met with success. It is known that cannabis extract has an effect on inflammatory disorders but the composition of active ingredients, inactive ingredients and possible harmful ingredients has not been established. One example of an inflammatory disease which we chose for this study is rheumatic disease.

9. The experimental approach involved the following steps: (a) extracting different variations of cannabis using different extraction methods (such as, for example, hot extraction, cold extraction and determination of the composition of extraction liquid) to provide different concentration of components in the extracts; (b) applying the extracts to different assays using different concentrations of components in the extracts; (c) measuring the effect of the extracts on the assayed biological phenomena using metabolomics based on LC-MS in both positive and negative mode; (d) using analytical techniques to determine the composition of the

extracts; (e) relating the chemical composition of the extracts to the measured effects in the assays using multivariate regression techniques (PLS); (f) determining, using variable selection, which chemical compounds relate to the measured effects in an assay; (g) chemically identify the compounds of the extracts which have a correlation with the measured effects in the assay; and (h) performing biological interpretation to determine what the underlying mechanism is for the measured effect in the assay.

10. The data analytical technique used in this research is called PLS (partial least squares), which is a multivariate regression technique. This technique is commonly used when interaction effects are to be expected (i.e., synergistic effect in this case; the effects of one compound is dependent upon the presence of other compounds). While many compounds are measured using the LC-MS technique, a second technique called variable selection is added to the model estimations. This variable selection technique allows for determining which of the variables (chemical compounds) have a significant effect on the measured effect.

11. Experimental details related to the key procedure of identification compounds from cannabis that are related to biological activity are described below for the two metabolomics procedures used; negative and positive ion mode.

12. The procedure for the detection of synergetic active compounds by positive ion mode metabolomics is described in detail below. As described above, LC-MS

data of cannabis extracts obtained in the positive mode were correlated to the selected bioassay results using PLS models. From this exercise, several peaks in the LC-MS data significantly correlated with the bioassay results (see Table 1). Of the seven correlating peaks, three could be chemically identified as AcCBD, CBG and CBC. Four other compounds remained unidentified. Using high-resolution MS, we tried to elucidate the elemental composition of these unknown peaks using the exact mass. MS/MS experiments were performed to identify the compounds. Firstly, some of the cannabis extracts, including the new cannabis extract, were analyzed by LC-MS to verify whether the correlating compounds were still present. In every extract, all compounds in Table 1 could be detected. The exact mass of AcCBD, CBG and CBC resulted in an elemental composition that was in agreement with the structural formula of these compounds. For the peak with m/z 377, it was observed that the highest m/z value of this peak was 375.2166 corresponding to $C_{22}H_{31}O_5$. This elemental composition corresponds well with that of either hydroxy-AcCBD or hydroxyl-THC-acid. Two peaks with the same exact mass are visible in the extract of CBD1-4 and also in the standard solution of cannibinoids CBG, CBD, CBC, CBN, THC, AcCBG, AcCBD and THC-acid. In the standard solution, the two peaks are of equal height while, in the extract, one peak is dominating. Based on the retention time and the relevant peak areas of this peak in the extracts compared to the concentration of cannabinoids in

the same extracts, this peak could be identified as hydroxyl-THC-acid. The peak with m/z 203 could not be detected with the FT-MS due to its low abundance. For the peaks with m/z 219 and 262, an elemental composition could be determined to be $C_{15}H_{32}O_1$ and $C_{17}H_{25}O_1$, respectively. Database searching using SciFinder resulted in about 1300 possible structures. Hence, it was not possible to identify these compounds.

13. Table 1: Results of identification of correlating compounds detected in the positive ionization mode

m/z	t_r (min)	Identity	Exact mass	Elemental composition	Remark
377	14.8	OH-THC-acid	377.2322	$C_{22}H_{33}O_5$ $[M+H]^+$	Highest m/z 375.2166 $C_{22}H_{31}O_5$ $[M+H]^+$
219	19.3	?	219.1742	$C_{15}H_{23}O_1$ $[M+H]^+$	
203	21.2	?	-	-	No peak was detected in FT-MS due to low abundance of this peak
359	23.4	AcCBD	359.2216	$C_{22}H_{31}O_4$ $[M+H]^+$	
262	23.7	?	262.2165	$C_{17}H_{28}O_1N_1$ $[M+NH_4]^+$	$C_{17}H_{25}O_1$ $[M+H]^+$
317	24.0	CBG	317.2475	$C_{21}H_{33}O_2$ $[M+H]^+$	
315	29.0	CBC	315.2318	$C_{21}H_{31}O_2$ $[M+H]^+$	

14. In a parallel procedure, we performed experiments to detect synergistic active compounds by negative ion mode metabolomics. The LC-MS data obtained in the negative ionization mode was also correlated to the selected bioassay results using PLS. Looking only at correlations with weighted importance > 90% resulted in about 32 relevant hits, i.e. mass.retention times. Further investigation of these hits, i.e. deleting noise and combining mass.retention times belonging to the same peak, i.e. compound, resulted in 12 relevant peaks (Table 2). Three peaks were tentatively identified as hydroxyl-cannabinoids based on the elemental composition and retention times. The elemental composition of the remaining three peaks, i.e. 371, 279 and 255, showed similarity with those of cannabinoids. However, database searching using SciFinder resulted in more than 1000 possible structures and therefore these peaks could not be conclusively identified.

15. Table 2: Results of identification of correlating compounds detected in the negative ionization mode

m/z	t_r (min)	Elemental composition $[M\text{-}H]^-$	(Tentative) Identity	Remarks
371.1858	18.3	$C_{22}H_{27}O_5$	?	
375.2171	20.1	$C_{22}H_{31}O_5$	hydroxy-AcCBG	
357.2065	22.1	$C_{22}H_{29}O_4$	AcCBD	-

359.2221	22.3	$C_{22}H_{31}O_4$	AcCBG	-
347.2260	22.6	$C_{21}H_{31}O_4$	dihydroxy-CBG	MS/MS shows two times loss of H_2O
357.2066	24.2	$C_{22}H_{29}O_4$	THC-acid	-
331.2277	24.7	$C_{21}H_{31}O_3$	hydroxy-CBG	MS/MS shows two times loss of H_2O
315.2330	25.6	$C_{21}H_{31}O_2$	CBG	Highest m/z corresponds to $[M-H+CH3COOH]^-$
313.2172	25.9	$C_{21}H_{29}O_2$	CBD	Highest m/z corresponds to $[M-H+CH3COOH]^-$
313.2174	29.7	$C_{21}H_{29}O_2$	CBC	Highest m/z corresponds to $[M-H+CH3COOH]^-$
279.2330	31.0	$C_{18}H_{31}O_2$	?	
255.2331	35.7	$C_{16}H_{31}O_2$	?	

16. The results of this research, as shown above, show that a number of extracts have a significant effect on the bioassay. Specific bioassays which were used to determine these effects is the assessment of inflammation of monocytes including (1) TNF-induced inflammation and (2) LPS-induced inflammation. These inflammations can be monitored directly or via inflammation markers which include, at least, the markers used in this study which include NF-kB; IL1, IL6, IL8, TNF-a, and PGE2. The multivariate technique used to correlate the bioassay data with the metabolomics data (both positive and negative ion mode) belongs to the so-called multivariate regression techniques in particular Partial Linear

Squares (PLS). Our results indicate that compounds originating from the cannabis plants and extracted during the extraction procedure have a synergistic effect. Using the novel approach claimed in this application, it is possible to trace and identify those components (i.e., compounds in Tables 1 and 2) among a multitude of other components in the plant extract which have little or no biological effect in our assays.

17. The undersigned declares that all statements made herein of my personal knowledge are true and that all statements made on information and belief are believed true; and further that these statements were made with the knowledge that any willful false statements are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that willful false statements may jeopardize the validity of this patent application or any patent issuing thereon.

Date: Driebergen, April 12, 2011

Dr. Jan van der Greef